EXHIBIT E

Heterogeneity of hematopoietic stem cells

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Hematopoietic stem cells are capable of multi-lineage differentiation to all blood cell types as well as self-renewal and radioprotection. Thy-1.1^{lo} Lin$^{-/lo}$ Sca-1^{+} cells are a heterogeneous mixture of quiescent and self-renewing hematopoietic stem cells as well as multi-lineage expanding cells.

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Introduction

Hematopoietic stem cells (HSCs) have both the ability to perpetuate themselves (self-renewal) and to differentiate to all blood cell lineages. Hematopoiesis is arranged in an irreversible descending hierarchy: it starts with clonogenic HSCs, passes through several stages of differentiation, and finally produces functionally mature blood cells, including erythrocytes, megakaryocytes (platelets), granulocytes, monocytes, macrophages, mast cells and the several classes of lymphocytes. In normal mice, most HSCs are not contributing to 'active' hematopoiesis at the time of isolation. However, after being injected into lethally irradiated mice, the isolated HSCs home to appropriate hematopoietic microenvironments and therein undergo massive expansion and differentiation to produce myeloerythroid cells for short-term rescue of the hosts (radioprotection), while simultaneously replenishing the HSC reservoir and maintaining steady-state hematopoiesis and lymphopoiesis.

In this review, we focus on recent studies revealing the heterogeneity of HSCs. Of course, the definition of HSCs can vary with the assays used. The adoptive transfer of hematopoietic cells to lethally irradiated hosts permits the quantitative analysis of both radioprotection and short- and long-term multi-lineage reconstitution (LTMR) of all blood cell subsets by donor-derived cells. Assays for clonogenic hematopoietic progenitors that may include HSCs exist for *in vitro* myeloerythroid progenitors colony forming unit-spleen (CFU-S) [1], thymic progenitors colony forming unit-thymus (CFU-T) [2,3], and cells that can initiate and maintain long-term hematopoiesis on stroma cells [long-term culture initiating cells (LTC-IC)] of the B-cell lineage under Whitlock–Witte conditions [4], or myeloerythroid cells under Dexter conditions [5•,6•]. Excellent reviews on HSC purification and assays for mouse HSCs [7–10], and recent progress in human HSC characterization [11,12] have been published.

Phenotypically defining mouse HSCs

To investigate the heterogeneity of HSCs, it is crucial to isolate them and their precursors as a highly enriched population and to characterize their activities quantitatively at the clonogenic cell level. Candidate mouse HSC populations have been enriched from adult bone marrow (BM) [3,13,14] and fetal liver (FL) [15–18]. The use of separation techniques (reviewed in [7,8]) is based on differences in physical [density gradient centrifugation and counter-flow centrifugal elutriation (CCE)], cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye Rhodamine (Rh)123 and DNA-binding dye Hoechst 33342).

Although there is no monoclonal antibody available yet that exclusively recognizes HSCs, these cells have been enriched by multiple cell surface characteristics, such as expression of H-2 [19], c-kit [20•,21•], Thy-1 [3,19], AA4.1 [22,23•], and stem cell antigen [3] (Sca-1, Ly6A/E [24,25]), or binding to wheat germ agglutinin (WGA) [13,20•,26,27]. By the combination of Thy-1.1 antigen expression at significant but low levels, the absence or very low expression of the lineage markers TER-119 (erythroid), B220 (B cell), Mac-1 (monocyte), Gr-1 (granulocyte), CD3, CD4 and CD8 (T cell), and high Sca-1 expression (Thy-1.1^{lo} Lin^{-} Sca-1^{+}), cell populations have been isolated from adult BM that were highly enriched in multipotent HSCs [3,18]. This rare subset of BM cells (0.05 per cent) contained clonogenic cells for each of the lineage outcomes, including LTC-IC [5], late CFU-S (1 in 10 cells injected), CFU-T (1 in 5 cells injected),

Abbreviations

BM—bone marrow; **CCE**—counter-flow centrifugal elutriation; **CFU-S**—colony forming unit spleen; **CFU-T**—colony forming unit thymus; **FACS**—fluorescence activated cell sorter; **FL**—fetal liver; **FR**—fraction; **HSC**—hematopoietic stem cell; **LTMR**—long-term multi-lineage reconstitution; **LTC-IC**—long-term culture initiating cells; **MDR**—multidrug resistance; **Rh**—Rhodamine; **R/O**—rotor-off; **WGA**—wheat germ agglutinin.

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and was enriched for radioprotection by a factor of 2000-fold resulting in LTMR [3,28]. Therefore, the initial study [3], from our laboratory, concluded that the phenotypically homogeneous Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cell population is highly enriched for HSC activity. Although it was not claimed that these cells were a population of homogeneous HSCs [3], several reports followed that assumed we had made that claim and critiqued it [29–31]. In fact, as described here and elsewhere [18,28,32–34], these Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ HSCs are heterogeneous by a large number of independent criteria .

Recently, low levels CD4 of antigen were detected on HSCs [35•]. It has been shown that intrathymic precursors also express low levels of CD4 and Thy-1.1 and high levels of H-2K and Pgp-1 [36]. To characterize low levels of expression of a given lineage marker, both staining reagents and the fluorescence activated cell sorter (FACS) instrument must be optimal to separate low levels of staining from background and high levels of staining. For these reasons, it has not yet been determined which lineage markers are expressed at low levels or are negative within the Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ population. (Lin$^-$ will therefore include Lin$^-$ and Lin^{lo} cells.)

It is important to exclude the possibility that cell populations other than phenotypically defined 'candidate' populations contain HSCs or their progenitors. To search for HSC activity in all BM fractions, Thy-1.1$^+$ versus Thy-1.1$^-$, Lin$^{-/lo}$ versus Lin$^+$, and Sca-1$^-$ versus Sca-1$^+$ cells were injected alone to detect radioprotection and LTMR, or together with 10^5 congenic BM cells to detect LTMR independent of radioprotection (which should reveal pre-HSCs). Thy-1.1$^-$ cells (representing 96 per cent of BM cells), Sca-1$^-$ cells (94–95 per cent) and Lin$^+$ cells (82–89 per cent), which together represent 99.95 per cent of BM cells, neither radioprotected nor contributed to LTMR. Therefore, Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells, but no other cells, appear to be the only pluripotent HSCs in adult C57BL/Ka-Thy-1.1 BM [37•].

Heterogeneity of HSCs during ontogeny

Heterogeneity of differentiation potential exists between fetal and adult HSCs. In human erythroid differentiation, differential expression of β-like globin genes is regulated by the locus control region, which is located 5′ of the ε-globin genes [38] and by the erythroid-specific DNA-binding protein GATA-1 [39] (reviewed in [40]). In mouse B-cell development, FL cells are better able than adult BM cells to give rise to B-1α cells (formerly called CD5$^+$ B cells) [41,42] (reviewed in [43]).

During mouse fetal life, the successive waves of T-cell receptor gene rearrangements and expressions are developmentally regulated (reviewed in [44–46]). Ikuta [46] showed that fetal, but not adult mouse HSCs have the capacity to differentiate into $V_\gamma3^+$ and $V_\gamma4^+$ T cells in fetal but not adult thymic microenvironments [18,33]. Early (day 14) FL precursors predominantly give rise to thymocytes with canonical $V_\gamma4$-$J_\gamma1$ transcripts, whereas late (day 18) and adult BM precursors predominantly give rise to $V_\gamma4$ thymocytes with modified $V_\gamma4$-$J_\gamma1$ junctions [33]. Thus FL HSCs have a different developmental potential from adult BM HSCs.

Heterogeneity of adult bone marrow HSCs

A mitochondrial-binding dye Rh123 has been useful in separating BM progenitor populations [47–50]. Chaudhary and Roninson [51•] suggest that low levels of Rh123 staining of hematopoietic cells correlate with its export due to expression of the product of the multidrug resistance (MDR)1 gene P-glycoprotein. Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells can be further divided into Rh123^{lo} and Rh123^{hi} cell types [32]. Although Rh123^{hi} Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells were highly enriched for day 13 CFU-S activity (105 cells with CFU-S activity per 1000 cells injected), they were not correspondingly enriched for pre-CFU-S activity [32]. The Rh123^{lo} Thy-1^{lo} Lin$^-$ Sca-1$^+$ subset was also highly enriched for day 13 CFU-S activity (23 cells with CFU-S activity per 1000 cells injected). Most striking was the observation that the Rh123^{lo} subset was most highly enriched for pre-CFU-S and LTMR activities, as tested by secondary BM or spleen cell transfer into lethally irradiated mice [32]. Li and Johnson [52••] recently showed that in response to combinations of growth factors, Rh123$^{mid/hi}$ Lin$^-$ Sca-1$^+$ cells produced more *in vitro* colony forming cells than Rh123^{lo} Lin$^-$ Sca-1$^+$ cells. Both 100 Rh123^{lo} and 100 Rh123$^{mid/hi}$ Lin$^-$ Sca-1$^+$ cells were equally radioprotective (90 per cent survival in 30 days). However, 100 Rh123^{lo} Lin$^-$ Sca-1$^+$ cells gave rise to 73 per cent of the donor-derived peripheral blood nucleated cells, whereas 100 Rh123$^{mid/hi}$ Lin$^-$ Sca-1$^+$ cells only contributed 0.8 per cent of the donor-derived peripheral blood leukocytes when tested at 19 weeks [52••].

Can radioprotection be separated from long-term reconstitution? Jones *et al.* [30] used CCE to separate BM cells into four fractions on the basis of size and density. The smallest cells that eluted at 25 ml min^{-1} [fraction (FR) 25] had undetectable levels of day 12 CFU-S activity and were not radioprotective at a dose of 1×10^5 cells. One fraction that was highly enriched for blast cell types (called Rotor-off, [R/O]), contained day 12 CFU-S activity and could radioprotect 15 out of 28 animals given 2×10^4 cells for at least 60 days. When 2×10^4 FR 25 cells were injected into irradiated syngeneic mice with 2×10^4 R/O cells, only FR 25 cells appeared to contribute to LTMR. Jones *et al.* [30] concluded that two independent classes of BM cells mediate two different phases of hematopoietic recovery in lethally irradiated animals: R/O cells contained progenitors that provide radioprotection via rapid, but short-term engraftment, whereas FR 25 contained pre-CFU-S or 'true' HSCs that could not contribute to radioprotection by early myeloerythroid development, but contained cells that could produce LTMR. They showed that the day 12 CFU-S activity peaked in the FR 29 cell fraction, fell off somewhat in FR 33 cells, but

stayed roughly level in the R/O cell fraction. They did not report studies of the radioprotection and LTMR activity of FR 29 and FR 33 cells [30].

We sought to test their conclusions that FR 25 cells had LTMR but not CFU-S or radioprotective activities, whereas the R/O fraction contained CFU-S activity and was radioprotective but did not contain LTMR activity. We found that the peak number of Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells was in the FR 29 cell fraction with fivefold fewer Thy1.1^{lo} Lin$^-$ Sca-1$^+$ cells in the FR 25 cell fraction, which was about 40 per cent depleted [34]. Surprisingly, FR 25 Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells gave rise to day 13 CFU-S activity, which was enriched by 100-fold, as did FR 29 cells, which was enriched by 450-fold, and 100 FR 25 or FR 29 Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells were fully radioprotective and had LTMR activity [34]. R/O Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells also contained day 13 CFU-S activity (enriched by 500-fold); 300 of these cells were required for radioprotection and LTMR [34]. Most of the discrepancies suggested by Jones *et al.* [30] and Iscove [31] therefore can be accounted for by an uneven distribution of Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells in these eluted fractions, as predicted [53], although quantitative, assay-specific differences could be observed [30]. A most interesting heterogeneity seemed to be in the R/O fraction, which was quantitatively less radioprotective; Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells in the R/O fraction are mainly Rh123^{hi} and 30–40 per cent of them are in the S/G_2/M phases of the cell cycle. In contrast, nearly all Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells in FR 25, FR 29 and FR 33 are in the G_0/G_1 phases [34].

About 20 per cent of Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells from adult BM are in the S/G_2/M phases of the cell cycle (WH Fleming, EJ Alpern, N Uchida, K Ikuta, GJ Spangrude, IL Weissman, unpublished data). When 100 Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ S/G_2/M cells ($\geq$ 2n amount of DNA) were isolated by FACS following staining with the DNA-binding dye Hoechst 33342, they only provided radioprotection in 23–30 per cent of irradiated mice, whereas when 100 Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells with 2n amount of DNA were injected, 90 per cent radioprotection and full LTMR ensued (WH Fleming, EJ Alpern, N Uchida, K Ikuta, GJ Spangrude, IL Weissman, unpublished data).

Heterogeneity of *in vivo* clonal HSC differentiation

How can individual HSCs contribute to steady-state hematopoiesis in mice? Previous indirect studies suggested that about 20 HSCs contribute originally to hematopoiesis in normal mice [54]. The numbers of HSC clones contributing to blood cell production varied depending on the status of mice (normal or irradiated etc.) and was influenced by the number of HSCs injected [28,54,55••,56••].

Oligoclonality of steady-state hematopoiesis was supported by a series of retroviral marking studies [22,57–60] (reviewed in [61,62]). Neben *et al.* [55••] also demonstrated oligoclonal LTMR by using alloenzyme as a marker. HSCs were enriched for cells with blast characteristics and low Hoechst 33342 intensity. When mice were repopulated with 1000–3000 sorted cells, the number of clones giving rise to donor erythrocytes was high (over 1000 clones) at early intervals (4 weeks) after transplantation, but then decreased rapidly to stable levels (2–5 clones), which over the period from 12 to 36 weeks remained relatively constant. Harrison and Zhong [56••] recently reported that in their alloenzyme-marked transfers, 21 cells per 10^5 BM cells initially contribute to hematopoiesis after 3 weeks, falling to 1.4 cells per 10^5 BM cells after 12–14 weeks.

Smith *et al.* [28] estimated that the order of eight Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ stem cells initially contributed to hematopoiesis in lethally irradiated mice when a total of 105 HSCs were injected. In recent studies, we estimated that approximately one out of 22 Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells injected into lethally irradiated mice contributes to detectable levels of blood cell production *in vivo* [34]. Short-term homing of labeled Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells to bones is approximately 5 per cent of input labeled (E Lagasse, IL Weissman, WH Fleming, unpublished data). In these *in vivo* analyses of single clonogenic cells, both lymphoid and myeloid lineages always resulted. Three distinct multi-lineage outcomes were observed from these individual HSC reconstitutions *in vivo*: firstly, transitory myelopoiesis and lymphopoiesis in the early phase ($<$8 weeks) of repopulation; secondly, multi-lineage differentiation with transient myelopoiesis ($<$16–28 weeks); and thirdly multi-lineage repopulation with sustained myelopoiesis (25–30 per cent of reconstitutions). Several conclusions can be derived from this work: firstly, most, if not all Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells are potent multi-lineage progenitors; secondly, Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells are heterogeneous; thirdly, the dose of bone marrow cells that will protect 95–100 per cent of lethally irradiated mice is 200 000 cells and this probably results in one to three clones participating in long-term clonogenic repopulation [3]. (One out of 2000 BM cells are Thy-1.1^{lo} Lin$^-$ Sca-1$^+$, one out of 22 home to BM and read out as multi-lineage precursors, and one out of four of these are long term multi-lineage reconstitutions.)

Heterogeneity of HSCs among mouse strains

Spangrude and Brooks [63•] revealed HSC phenotype heterogeneity among mouse strains. They demonstrated that HSCs from Thy-1.1 strains were found in Thy-1.1^{lo} but not Thy-1.1$^-$ populations, whereas those from Thy-1.2 strains were found in both Thy-1.2^{lo} and Thy-1.2$^-$ populations. However, Thy-1 expression on T cells does not differ in these mice [63•]. Thus, there appears to be an allele-specific variability in Thy-1 expression in some cell subsets (HSCs) but not others (T cells). It is not yet clear at which level of gene expression the differential regulation operates.

Van Zant *et al.* [64••,65,66] have suggested that genetic differences might influence HSC behavior. They created allophenic mice by aggregating embryos of eight cells

from DBA/2 and C57BL/6 mice, and tested the genotypic composition of red and white cells in the blood of the allophenic chimeras for over 30 months [66]. DBA/2 blood cells tended to dominate for the first half of the life of chimeras, then C57BL/6 blood cells took over as the mice aged. Serial transplantations of BM cells from one allophenic chimera were performed. Strikingly, in both primary and secondary irradiated hosts, early engraftment was predominantly achieved by DBA/2 cells, while long-term engraftment was supported by C57BL/6 cells. It shall be important to understand the genetic control of HSC behavior, and whether it is intrinsic or extrinsic to the HSCs themselves.

A model of HSC heterogeneity

Heterogeneity exists among mouse HSCs in terms of size, cell cycle status, Rh123 staining, relative radioprotective effect and LTMR activity. One possible model to explain the data is shown in Fig. 1. In this view Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells can be divided into quiescent HSCs, self-renewing HSCs and dividing pluripotent cells that do not self-renew, but enter an expansion pool that itself gives rise to more differentiated progenitors. If one isolates all such progenitors simply by phenotypic markers (e.g. Thy-1.1^{lo} Lin$^-$ Sca-1$^+$), the HSCs would be a mixture of quiescent and self-renewing HSCs as well as expanding HSCs and/or progenitors. It should be noted that these changes could be intrinsic to HSCs or result from their passage through changing microenvironments.

Intrinsic changes in the HSC pool that may explain the heterogeneity could, for example, depend on the number of cell divisions that have occurred in the life history of each HSC, perhaps via restricting their profile of or response to cell surface receptors for cytokines or other ligands. The fate of individual HSCs might be determined by hematopoietic microenvironments. In the *in vivo* HSC transfer studies, injected HSCs have to circulate in the blood and seed hematopoietic microenvironments. Repopulation outcomes could depend on the specific microenvironments where individual HSCs land. For example, hematopoietic compartments were observed in the early phase of hematopoietic engraftment of lethally irradiated animals; the spleen was predominantly erythro-

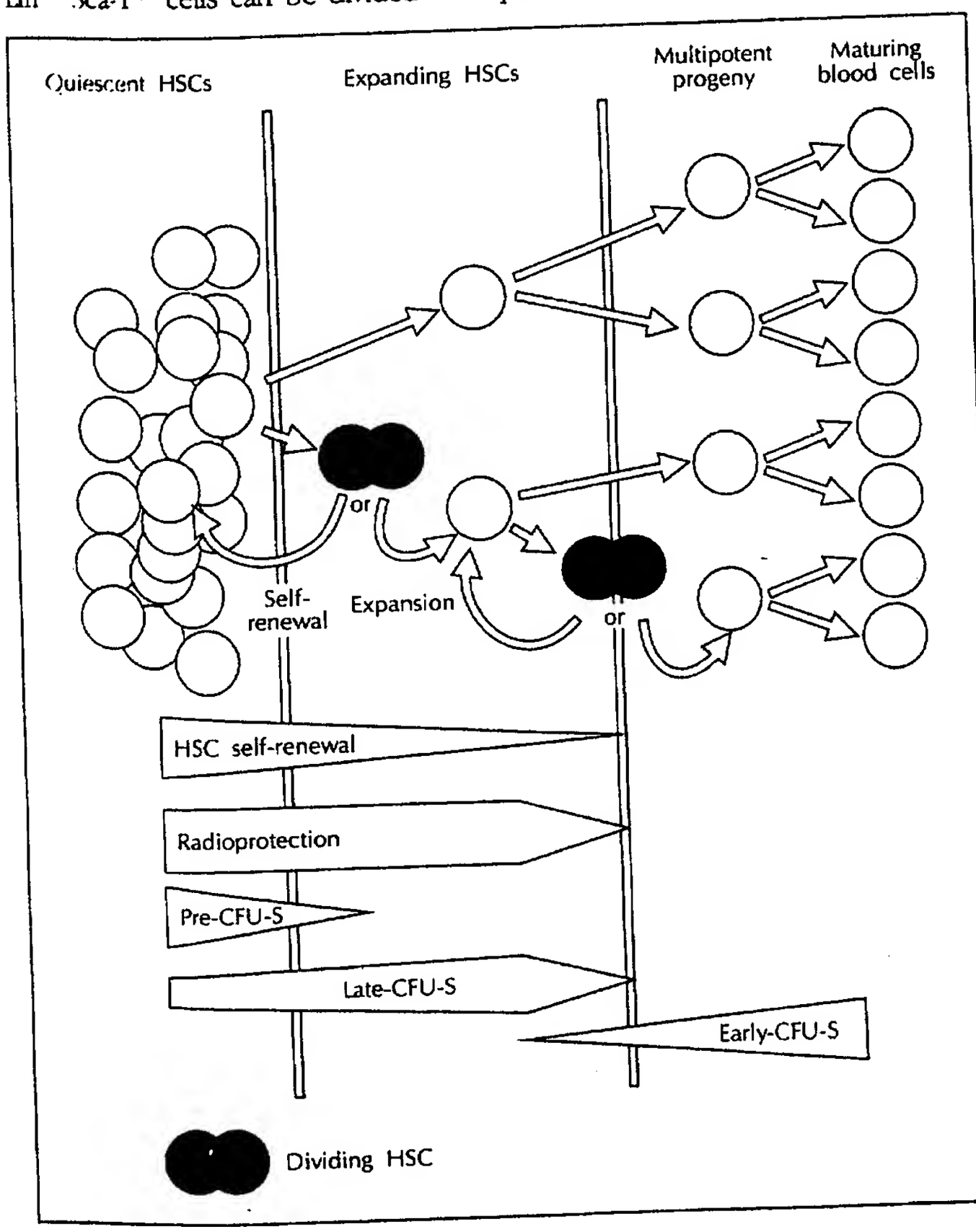

Fig. 1. A model of the early stages of hematopoiesis. In this model a highly enriched BM population isolated by phenotypic markers (e.g. Thy-1.1^{lo} Lin$^-$ Sca-1$^+$) contains cells that are mixture of quiescent, self-renewing and expanding HSCs, as well as multipotent progenitors. In normal mice, most HSCs are quiescent: quiescent HSCs begin to divide, and give rise to HSC progeny that are either capable of self-renewal to the quiescent pool or proceed to the expansion pool; some of the cells in the expansion pool continue to divide and enter the multipotent progeny and maturation pools. The represented HSC activities are tested by injecting hematopoietic cells into lethally irradiated mice. Therefore, one can not exclude the possibility that HSCs have different behaviours in normal mice. Intrinsic changes in the HSC pool that may explain the heterogeneity could, for example, depend on the number of cell divisions that have occurred in the life history of each HSC, which perhaps restricts their profile of, or response to, cell surface receptors. The frequency and distribution of renewing versus expanding HSCs could be regulated by hematopoietic microenvironments (see Fig. 2). The relative probability of cells possessing the described hematopoietic precursor activities at each stage of development is shown in the lower half of the figure.

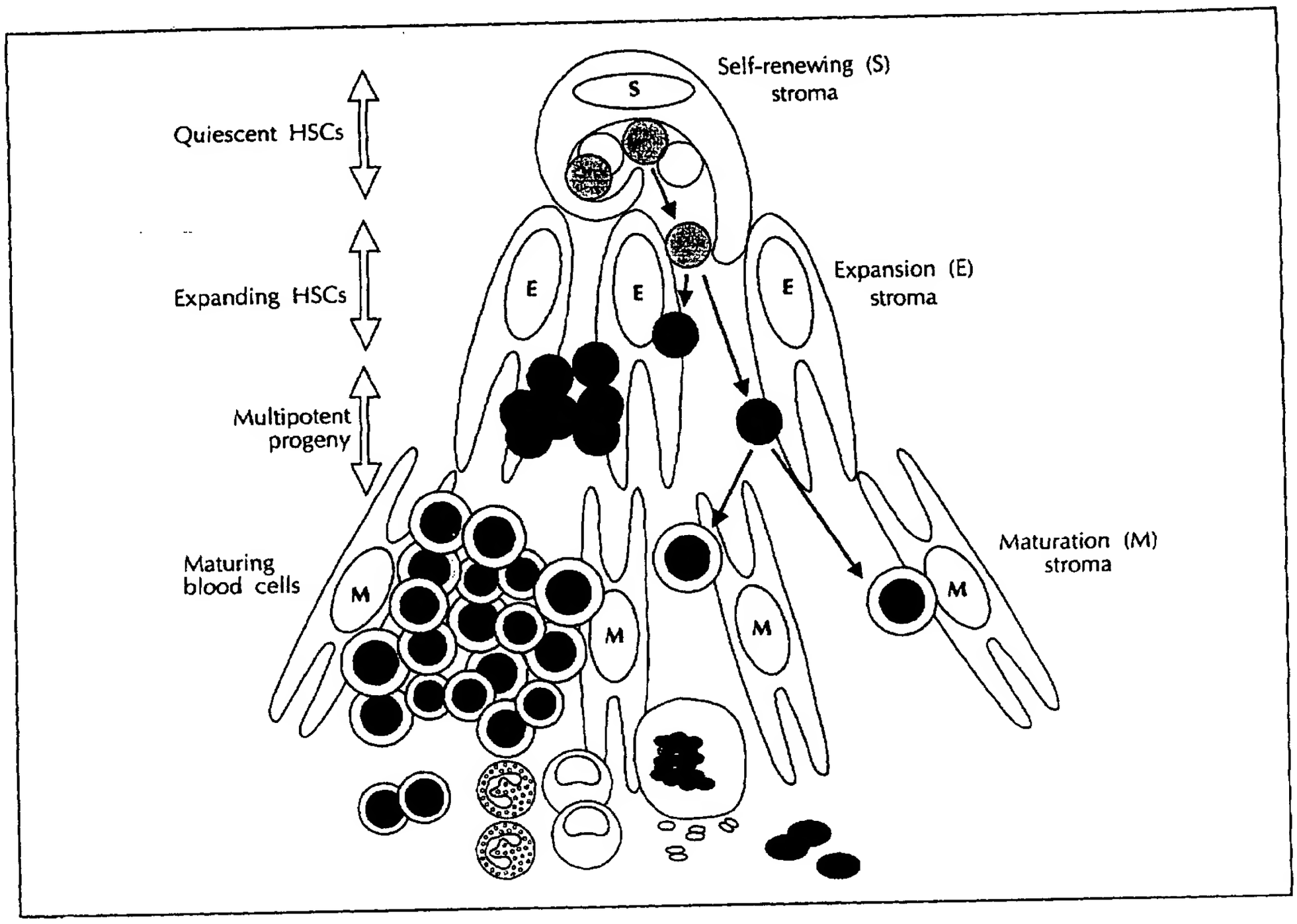

Fig. 2. A model of microenvironmental determination of HSC heterogeneity. In this model HSCs are subdivided into functional subsets by the microenvironments in which they reside. In this view, the HSCs residing in the self-renewing (S) stroma occupy a physically limited niche. When a HSC in this niche undergoes cell division, not all of its progeny HSCs may occupy the S niche any longer but are pushed into the next niche, bordered by expansion (E) stroma. Although HSCs in both S and E niches could express the same profile of interaction receptors, the S and E stroma could provide different stimuli to HSCs. For example, in this model, the S stroma would promote self-renewal of HSCs, whereas E stroma provides microenvironments for extensive proliferation of HSCs without (or with less extensive) self-renewal. As proliferation in the E niches occurs, they too would become filled; their daughter cells would enter the fields wherein maturation occurs, presumably governed by maturation (M) stroma. The number of available S niches would presumably regulate the number of injected HSCs that can engraft and expand in the context of bone marrow transplantation. Reproduced with permission from [70].

genic, whereas the BM was predominantly myelogenic [67]. It has been suggested that HSC activity is localized to the endosteal region [68,69]. When we examined BM sections 7 days after irradiation and HSC transplantation, distinct foci of hematopoiesis were localized mainly in proximity to the endosteum [34]. If there are a limited number of endosteal sites for HSC seeding, saturation of these sites could limit the number of 'true' HSCs that respond.

Because the fate of HSCs may be determined by particular stroma that interact with the HSCs, it is not inconceivable that several classes of specific stromal microenvironments may exist, and influence the fate of HSCs after cell division by cell–cell contacts and/or cytokines. A model of such compartmentalization is shown in Fig. 2. It is important to study the existence, location, biology and biochemistry of such stromal microenvironments.

Conclusion

Mouse Thy-1^{lo} Lin-1^{lo} Sca-1^{+} cells include HSCs and multilineage (T cell, B cell, myeloerythroid) progenitors; the latter population differs from the former on the basis of their probability of supporting sustained myelopoiesis as well as HSC self-renewal. In adult mice, HSCs with greater self-renewal capacity tend to be small, non-dividing cells that stain at low levels with Rh123, whereas the HSC/multilineage progenitors with lower self-renewal capactiy tend to be larger cells, more likely to be in the cell division cycle, and stain at higher levels with Rh123. In addition, the development potential of fetal HSCs, is significantly broader than that of adult HSCs. It shall be important to determine the lineage relationships between these heterogeneous HSCs, the extent to which their developmental clocks are intrinsic versus dependent on the

microenvironment, and the genetic programs that control and/or mediate these developmental choices.

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